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Production and Degradation of Oxalic Acid by Brown Rot Fungi

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Our results show that all of the brown rot fungi tested produce oxalic acid in liquid as well as in semisolid cultures. *Gloeophyllum trabeum*, which accumulates the lowest amount of oxalic acid during decay of pine holocellulose, showed the highest polysaccharide-depolymerizing activity. Semisolid cultures inoculated with this fungus rapidly converted ^{14}C-labeled oxalic acid to CO_2 during cellulose depolymerization. The other brown rot fungi also oxidized ^{14}C-labeled oxalic acid, although less rapidly. In contrast, semisolid cultures inoculated with the white rot fungus *Coriolus versicolor* did not significantly catabolize the acid and did not depolymerize the holocellulose during decay. Semisolid cultures of *G. trabeum* amended with desferrioxamine, a specific iron-chelating agent, were unable to lower the degree of polymerization of cellulose or to oxidize ^{14}C-labeled oxalic acid to the extent or at the rate that control cultures did. These results suggest that both iron and oxalic acid are involved in cellulose depolymerization by brown rot fungi.

One of the most destructive forms of wood decay is caused by fungi that cause brown rots. During the early stages of colonization, fungal hyphae invade wood cells and secrete agents that bring about a rapid decrease in the number of glucosyl residues per cellulose chain, from 10,000 to 15,000 to about 200 (6, 7). The latter corresponds to the average size of cellulose crystallites (11).

Highley (17) has reported that an oxidative system is involved in cellulose depolymerization by brown rot fungi, showing that there was a significant increase of carbonyl and carboxyl groups in the cellulose during decay. Since most of the pore sizes in sound wood are too small to allow cellulolytic enzymes to penetrate the wood, only low-molecular-weight molecules could be responsible for this depolymerization (7, 12). The current working hypothesis on the nature of the depolymerizing agent is that oxygen-derived radical species are responsible for the initial attack of the cellulose polymer. Indeed, Halliwell (16), and Kirk et al. (21) have shown that Fenton's reaction [a mixture of Fe(II) and H_2O_2, which is known to generate ·OH radicals] causes a degradation of cellulose similar to that resulting from brown rot decay. Koenigs (23) demonstrated that H_2O_2 is secreted by brown rot fungi and that wood contains enough iron ions [as Fe(III)] to make this hypothesis feasible. Moreover, Gutteridge (13) found that iron(III) salts in the presence of a chelator (EDTA) and a superoxide-generating system could oxidize several neutral sugars in reactions dependent on the reduction of Fe(III) to Fe(II) by superoxide.

Oxalic acid also has been implicated in the wood-decaying system of these fungi. Takao (34) demonstrated that brown rot fungi secrete significant amounts of oxalic acid in liquid cultures, whereas white rot fungi apparently do not. The latter was attributed to the presence of an intracellular oxalate decarboxylase in white rot fungi which decomposes oxalate to CO_2 and formate. This enzyme could not be detected in cultures of brown rot fungi (32). Later, Schmidt et al. (31) demonstrated in vitro that oxalic acid could reduce Fe(III), normally present in wood, to Fe(II), with oxalic acid simultaneously oxidized to CO_2. Furthermore, this iron reduction significantly increased the cellulose-depolymerizing activity of an iron-H_2O_2 system.

However, the role of oxalic acid in wood decay by brown rot fungi in vivo is not fully understood. Koenigs (23) demonstrated that isolates of *Gloeophyllum trabeum* that caused extensive decay lowered the pH of wood, whereas those that resulted in low amounts of decay did not. In this regard, Agosin et al. (1) showed that the acidic conditions developed by brown rot fungi are essential for wood decay. However, Micales and Highley (26) could not establish a correlation between the wood-decaying ability of various isolates of *Poria placenta* (*Postia placenta*) and their oxalic acid production.

The importance of oxalic acid during the invasion of host tissue by phytopathogenic fungi has been reported previously (5, 24, 25, 33). It has been suggested that the oxalic acid produced by *Sclerotium rolfsii* during pathogenesis acts synergistically with endopolygalacturonase, lowering the pH of the infected tissues to a level optimal for the activity of this enzyme. Furthermore, the calcium present in structural pectates can be strongly chelated by oxalic acid. As a consequence, plant tissues are rendered more susceptible to invasion by this fungus (29).

The aim of the present work, therefore, was to investigate further the possible role of oxalic acid in the cellulose depolymerizing system of brown rot fungi. First, the production of oxalic acid by various brown rot fungi under liquid and semisolid culture conditions was studied, and its relationship with cellulose depolymerization was assessed. Then, ^{14}C-oxalic acid was added at different stages of wood decay to determine the relationship between CO_2 production and cellulose depolymerization. Finally, to assess the participation of iron salts in the depolymerization of cellulose by these fungi, semisolid fungal cultures were incubated in the presence of the siderophore desferrioxamine (14) and the fate of ^{14}C-oxalic acid was followed.

MATERIALS AND METHODS

Fungi. The brown rot fungi *P. placenta* (MAD-698), *G. trabeum* (MAD 617-R), and *Wolfiporia cocos* (FP 90850-R) and the white rot fungus *Coriolus versicolor* (*Trametes versicolor*) (INTA 9A) were used in these experiments.

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These fungi were maintained in malt-yeast extract agar slants at 28°C. Inocula were grown in agitated liquid cultures, and the resulting mycelial pellets were centrifuged, washed, and ground before inoculation, as described elsewhere (2).

Liquid cultures. Static liquid cultures were conducted in 100-ml Erlenmeyer flasks with 10 ml of mineral medium (20) containing 55 mM glucose and 2.2 mM ammonium tartrate as carbon and nitrogen sources, respectively. The medium was buffered with 50 mM sodium 2,2-dimethylsuccinate, pH 4.50.

Semisolid cultures. (i) Cultivation methods. All decay studies were performed in specially designed glass columns (30) which were filled with 1.2 g of pine (*Pinus radiata*) holocellulose, prepared by the method of Timell (35), and humidified to a final moisture content of 75% (wt/wt) with a mineral medium containing the inoculum (20). During incubation at 28°C, the columns were constantly oxygenated through the bottom with humidified air at a flow rate of 3.2 ml/min.

(ii) Radiolabeling studies. ^{14}C-oxalic acid, 5.55×10^5 dpm, (50 μl), was fed to semisolid cultures after 0, 7, and 30 days of decay by applying it with a 50-μl Hamilton syringe to the batch at several sites. Decarboxylation of ^{14}C-oxalic acid was followed after trapping $^{14}CO_2$ from the outlet air flow column in 10 ml of 2-phenylethylamine scintillation solution, as described by Kadam and Drew (19). All experiments were conducted in at least four replicates.

To study the role of iron in the breakdown of oxalic acid, desferrioxamine (Desferal; Ciba-Geigy, Basel, Switzerland), a specific iron-chelating agent, was added to semisolid cultures at a concentration of 15 mg/g of holocellulose. After 7 days of decay, 50 μl of ^{14}C-oxalic acid was fed, and the evolution of $^{14}CO_2$ was followed. The amount of desferrioxamine added to the cultures was calculated considering the average iron content of pine wood (11).

Miscellaneous methods. The degree of polymerization (DP) of cellulose was determined viscosimetrically (6) after solubilization of holocellulose with cupriethylenediamine hydroxide solvent. Cupriethylenediamine was prepared by a standard procedure of the American Society for Testing and Materials (4).

The amount of free oxalic acid present in holocellulose-decayed samples was enzymatically determined on supernatants of thoroughly water-extracted material, using an oxalate kit (Sigma Chemical Co., St. Louis, Mo.). Calcium oxalate was solubilized by 0.2 N HCl from the water-extracted solid residue and determined as free oxalic acid as described above. For liquid cultures, oxalic acid was directly determined from an aliquot of the cell-free culture medium, using the same procedure.

The capacity of brown and white rot fungi to solubilize calcium oxalate was assayed in a semisolid agar culture medium containing 0.5% (wt/vol) calcium oxalate, 0.1% (wt/vol) glucose, and basal mineral salts. The pH of the medium was buffered as described above. Oxalate degraders were detected in this medium by the formation of clear zones around the fungal colonies (8).

The pH of the samples was determined by using an Orion pH recorder, model SA 720.

Mycelial growth in liquid cultures was determined gravimetrically (11).

Weight loss was followed by determining the amount of carbon lost as CO_2. For this purpose, CO_2 was continuously trapped during cultivation in 10 ml of 0.5 N NaOH and quantified every 2 days by titration of the residual NaOH with 0.2 N HCl (2).

Crude extracts were obtained from 1.2 g of holocellulose semisolid cultures decayed by *G. trabeum* for 7 days. The extraction process was done with 2.5 ml of water (three times), squeezing and soaking through a 0.2-μm-pore-size membrane filter. ^{14}C-oxalic acid, 10,000 dpm, was added to 1 ml of the water extracts, and the mixture was incubated for 24 h at 25°C. Oxalic acid oxidation was measured after trapping and counting the $^{14}CO_2$ produced.

RESULTS

Liquid cultures. (i) Production of oxalic acid by brown and white rot fungi. Oxalic acid was secreted into the culture medium from the first day of incubation and increased with growth (Fig. 1). *W. cocos* and *P. placenta* accumulated the highest amounts of oxalic acid, reaching 2.00 and 0.95 mg/ml (standard deviation, $<\pm 0.05$), respectively, after 4 days of cultivation. As a consequence, the pH of these fungal cultures rapidly dropped to around pH 3.0, even though the culture medium was initially buffered at pH 4.5 with 50 mM 2,2-sodium dimethylsuccinate. In contrast, the cultures inoculated with *G. trabeum* exhibited only a limited production of oxalic acid, reaching a maximum of 0.20 ± 0.04 mg/ml after 5 days of incubation. Finally, only trace amounts of the acid were detected in the cultures of the white rot fungus *C. versicolor*.

(ii) Oxalic acid and growth. Liquid cultures containing oxalic acid as the only carbon source showed limited, if any, growth, indicating that oxalate is not utilized after its secretion (data not shown). *C. versicolor* was the only species studied that was able to metabolize calcium oxalate crystals, as shown by its ability to produce clear zones in a calcium oxalate agar medium.

Semisolid cultures. (i) Oxalic acid production and cellulose depolymerization by brown and white rot fungi. Changes in the DP of holocellulose incubated with three brown rot fungi under semisolid culture conditions are illustrated in Fig. 2. *G. trabeum* showed the highest rate of depolymerization, reaching a leveling DP after 15 days of cultivation. In contrast, *C. versicolor*, a white rot fungus, caused only a very limited decrease in the DP of the holocellulose during decay, although an appreciable degradation of the cellulose occurred ($22.0\% \pm 0.5\%$ weight loss after 25 days of incubation). The holocellulose cultures of all three brown rot fungi rapidly dropped to pH values of around 3.0 after 3 to 5 days of cultivation and then remained constant. The DP decrease observed during the early phases of decay is inversely correlated with the increase in oxalic acid content of the semisolid cultures of *P. placenta* and *W. cocos*. Oxalic acid content decreased after the onset of cellulose depolymerization, however, reaching a plateau after 15 days of decay. Surprisingly, in cultures of *G. trabeum* oxalic acid content as well as calcium oxalate were low throughout the experiment (see Fig. 6, closed symbols), although this fungus showed the highest cellulose depolymerizing activity (Fig. 2). The lowering of pH by *G. trabeum* could be due to the production of other unidentified acidic compounds from the metabolism or catabolism of this fungus, as we detected by high-pressure liquid chromatography with an anionic exchange column (data not shown).

Finally, as expected, the white rot fungus *C. versicolor* did not lower the pH of the semisolid cultures, nor did it accumulate oxalic acid during decay.

(ii) Fate of ^{14}C-oxalic acid at different stages of cellulose depolymerization by brown and white rot fungi. The fate of radiolabeled oxalic acid during holocellulose decay is illus-

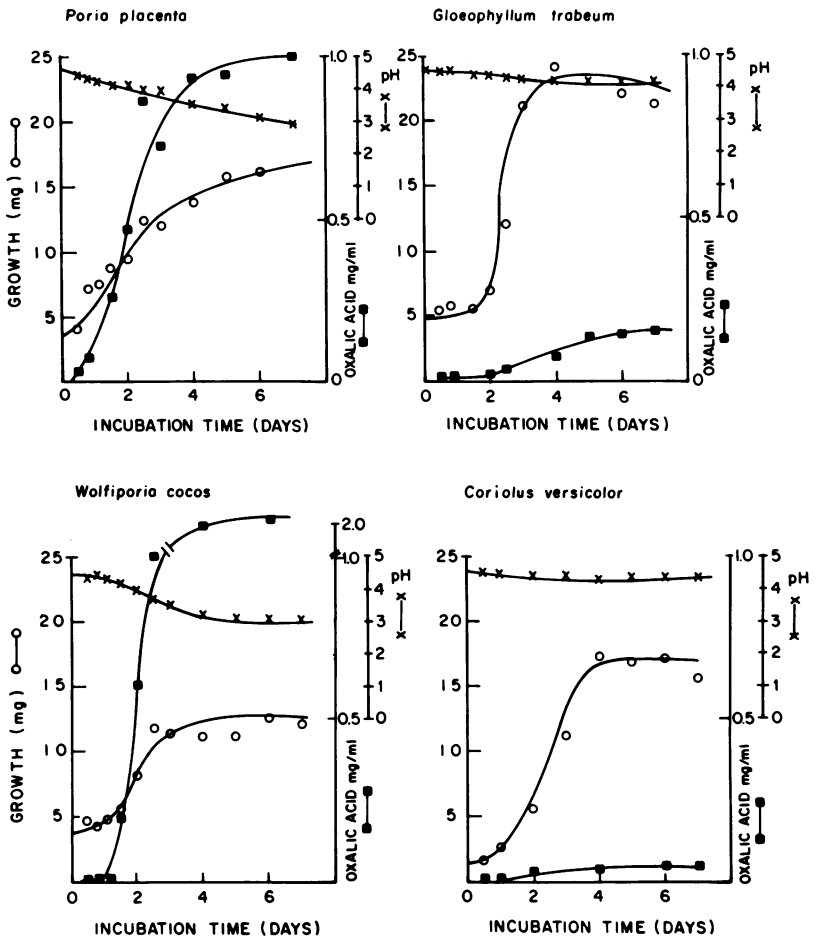

FIG. 1. Oxalic acid production in liquid cultures by the brown rot fungi *P. placenta*, *G. trabeum*, and *W. cocos*. The white rot fungus *C. versicolor* was used as a control. ×, pH; ■, oxalic acid; ○, growth.

trated in Table 1 and Fig. 3. When ^{14}C-oxalic acid was added at the beginning of incubation, *G. trabeum* and *P. placenta* generated $^{14}CO_2$ after lags of 2 and 4 days, reaching maxima of 85% ± 4% and 55% ± 6% mineralization of the oxalic acid added after 14 and 16 days of incubation, respectively.

When 7-day-old brown-rotted cultures were fed with the ^{14}C-oxalic acid, a very high mineralization rate was obtained, especially by *G. trabeum* (Table 1 and Fig. 3). Furthermore, no lag in the onset of $^{14}CO_2$ release was observed for either fungus. In contrast, *C. versicolor* showed only a limited ability for degradation of the radiolabeled substrate (Fig. 3). Although both brown rot fungi rapidly reached a maximum for $^{14}CO_2$ production, complete oxidation of the ^{14}C-oxalic acid added was not achieved.

Finally, when cellulose had already reached its lowest DP (about 200), i.e., after 30 days of degradation, the ability to oxidize ^{14}C-oxalic acid to $^{14}CO_2$ was still present, although to a reduced extent (Table 1).

(iii) Effect of desferrioxamine addition on the cellulose depolymerizing system of *G. trabeum*. Our assays showed that *G. trabeum* accumulates low quantities of oxalic acid but has a high cellulose depolymerizing activity and a high ability to oxidize oxalic acid to CO_2. The involvement of iron in the degradation of oxalic acid was examined by testing the action of the Fe(III) chelator desferrioxamine. The effect of desferrioxamine on cellulose depolymerization is shown in Fig. 4. A substantial inhibition of cellulose depolymerization was obtained in the presence of the siderophore. Desferrioxamine also caused a marked inhibition of $^{14}CO_2$ release from ^{14}C-oxalic acid (Fig. 5). Even though a lower weight loss (determined as carbon loss) was found for cultures containing desferrioxamine, a similar extent of decay in cultures without the iron chelator produced a leveling off of the DP of the cellulose component to about 200 (Fig. 4).

Figure 6 shows a significant increase in oxalate and oxalic acid content in cultures containing the iron chelator, suggesting that the low levels of oxalic acid observed in cultures of *G. trabeum* result from its consumption during cellulose depolymerization rather than from low production.

(iv) In vitro mineralization of ^{14}C-oxalic acid. We found that water extracts of 7-day-old pine holocellulose semisolid cultures, decayed by *G. trabeum*, could oxidize ^{14}C-oxalic acid up to 9%. This oxalic acid-oxidizing activity was destroyed when the extract was boiled for 30 min at 100°C.

DISCUSSION

The mechanism implicated in the depolymerization of cellulose during wood decay by brown rot fungi suggests the participation of an oxidative agent of low molecular weight. The current working hypothesis is that highly reactive oxygen-derived radicals are involved in this process. Hy-

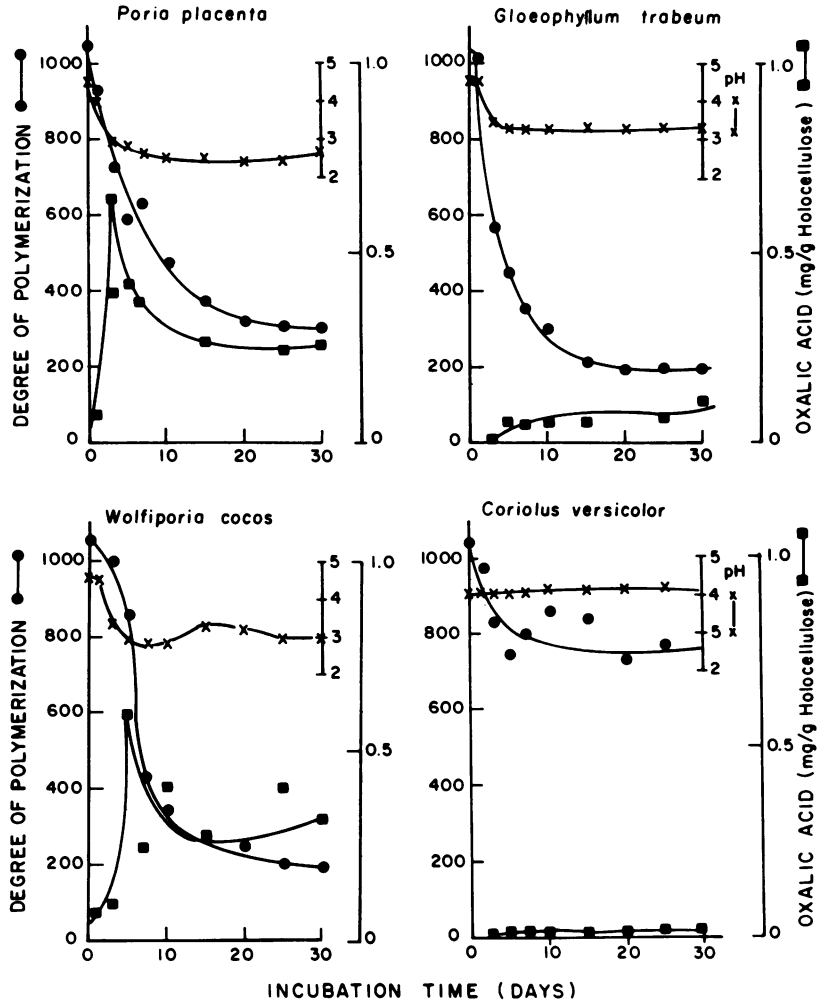

FIG. 2. Evolution of pH (×), oxalic acid (■), and cellulose depolymerization (○) during decay of pine holocellulose by wood-decaying fungi under semisolid culture conditions.

TABLE 1. Characteristics of mineralization rate of ^{14}C-oxalic acid during the decay of pine holocellulose by two brown rot fungi

Strain	Incubation time (days)[a]	Wt loss (%)[b]	Initial DP	$^{14}CO_2$ production		
				Lag (days)	Initial rate (% $^{14}CO_2$ h^{-1})[c]	Total ^{14}C (% total ^{14}C)[d]
G. trabeum	0		1,000	2	0.34	85
	7	3.2	390		28.00	80
	30	29.0	200		5.42	40
P. placenta	0		1,000	4	0.16	55
	7	3.2	500		1.91	65
	30	20.2	298		1.88	20

[a] Incubation time at which the ^{14}C-oxalic acid (50,000 dpm) was added to the cultures.

[b] Determined as CO_2 produced from the oxidation of holocellulose by the action of these fungi (as percentage of initial carbon substrate).

[c] $^{14}CO_2$ produced by the fungus from ^{14}C-oxalic acid (as percentage of total ^{14}C added).

[d] $^{14}CO_2$ recovered after 35 days of incubation.

droxyl radicals could be generated in wood by the Fenton reaction, $Fe(II) + H_2O_2 \rightarrow Fe(III) + \cdot OH + OH^-$. The resulting Fe(III) might be converted back to Fe(II) for further · OH generation by the oxalic acid secreted by this type of fungus (23).

Our results confirm previous work by Takao (34) concerning the production of oxalic acid by brown rot fungi during growth in liquid culture, and only trace amounts of oxalic acid could be found in white rot fungal cultures, presumably as a consequence of the presence of an intracellular oxalate decarboxylase produced by these fungi (32) or due to the involvement of oxalic acid in the lignin peroxidase system of *Phanerochaete chrysosporium*, as described recently by Akamatsu et al. (3) and Popp et al. (27).

Oxalic acid was also secreted by brown rot fungi during the decay of pine holocellulose under semisolid culture conditions. Our data indicate that oxalic acid production is associated with the drop in culture pH during the first stages of decay; moreover, oxalic acid concentration decreases in parallel to cellulose depolymerization. *G. trabeum*, which exhibited the highest rate of cellulose depolymerization, showed the lowest oxalic acid accumulation. Therefore, the drop of culture pH should be associated with other com-

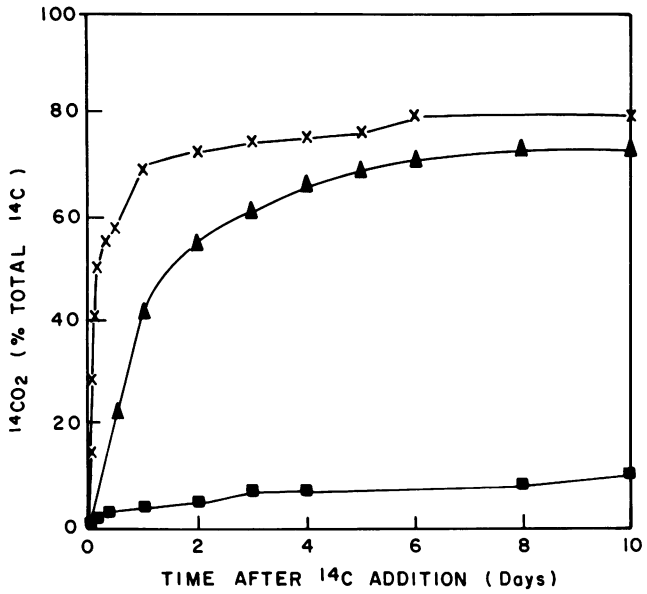

FIG. 3. Mineralization of ^{14}C-oxalic acid in semisolid cultures of pine holocellulose by the wood-decaying fungi *G. trabeum* (×), *P. placenta* (▲), and *C. versicolor* (■). ^{14}C label was added after 7 days of incubation.

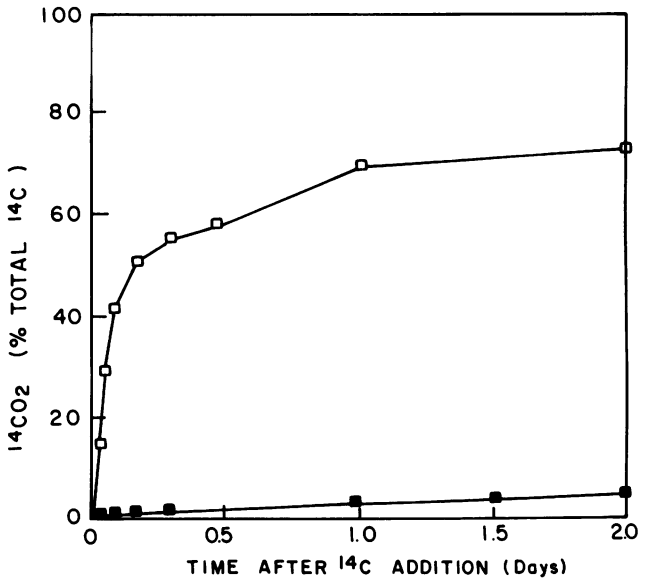

FIG. 5. Mineralization of ^{14}C-oxalic acid in semisolid cultures of pine holocellulose decayed by *G. trabeum* in the presence (■) or absence (□) of desferrioxamine. ^{14}C-oxalic acid was added after 7 days of incubation.

pounds, other organic acids produced by this fungus, and/or sugar acids from the catabolism of polysaccharides (21). Moreover, the onset of cellulose depolymerization in semisolid cultures inoculated with this fungus was found to occur at the beginning of cultivation. Our experiments with ^{14}C-oxalic acid clearly demonstrate that brown rot fungi possess the ability to oxidize oxalic acid at a very high rate, in contrast to previous reports by Takao (34), although the latter reports concerned liquid rather than semisolid cultures. *G. trabeum* showed the highest $^{14}CO_2$ release rate from ^{14}C-oxalic acid. This high oxidation rate may well explain the low amounts of oxalic acid detected in semisolid cultures as a result of the participation of oxalic acid in cellulose depolymerization. *P. placenta* showed a similar pattern of degradation. Finally, as expected, the white rot fungus *C. versicolor* showed a very limited ability to release $^{14}CO_2$ from ^{14}C-oxalic acid or to depolymerize holocellulose during decay.

Experiments conducted in the presence of the siderophore desferrioxamine indicated that sequestration of iron significantly inhibited cellulose depolymerization. At the same

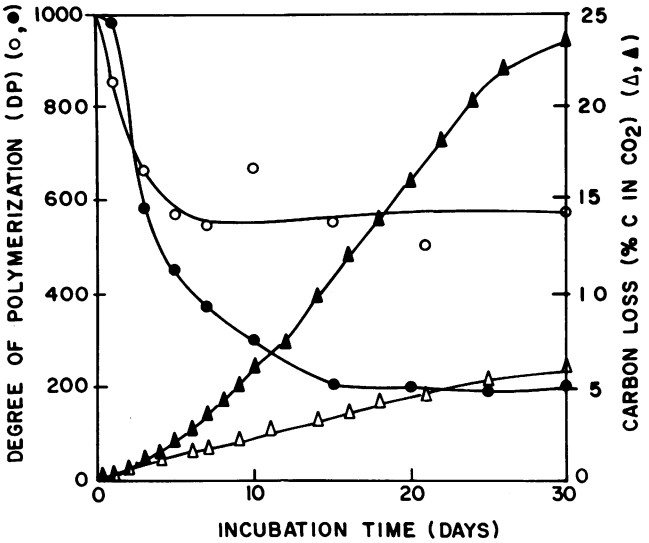

FIG. 4. Evolution of carbon loss (△, ▲), and DP (○, ●) during pine holocellulose degradation by the brown rot fungus *G. trabeum* in the presence (open symbols) or absence (closed symbols) of desferrioxamine.

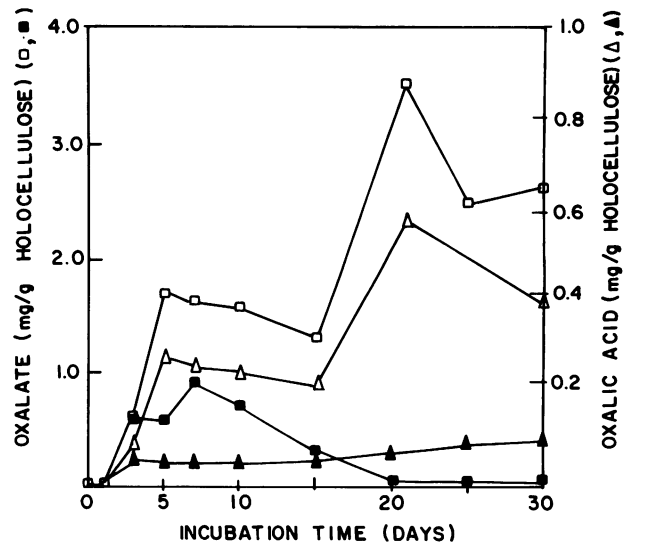

FIG. 6. Evolution of oxalate (□) and oxalic acid (△) during pine holocellulose degradation by the brown rot fungus *G. trabeum* in the presence (open symbols) or absence (closed symbols) of desferrioxamine. $P > 0.05$ by t test.

time, oxalic acid and oxalate concentrations were higher in the presence of desferrioxamine than in the control, and the production of $^{14}CO_2$ from ^{14}C-oxalic acid was decreased. Transition metals (including iron) accumulate during brown rot decay of cotton cellulose (22).

Overall, these results support the proposal that oxalic acid oxidation is iron dependent, presumably causing reduction of Fe(III) to Fe(II), and that the latter is effectively involved in cellulose depolymerization, perhaps by generating ·OH radicals in a Fenton reaction.

The Fenton reaction requires H_2O_2 (16, 21, 31). Detection of H_2O_2 in brown rot cultures has been difficult (18, 23, 36), perhaps because of its high consumption rate. In this respect, another possible explanation for the role of oxalic acid could be that there is an extracellular oxalate oxidase capable of both oxidizing oxalic acid and generating H_2O_2. Indeed, Enoki et al. (9) recently reported the presence of an extracellular one-electron oxidizing activity in wood-decaying cultures of brown rot fungi, but the ability of this enzymatic system to oxidize oxalic acid was not reported.

Hydroxyl radicals are highly reactive and combine with the molecules present at or very close to their site of formation (15, 28). If they are responsible for cellulose depolymerization, then they must be generated adjacent to the cellulose chains. The mechanism by which the required iron for this reaction is translocated to the sites where ·OH generation is required remains to be determined.

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